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Marker-assisted backcrossing: a practical example

M. RAGOT¹, M. BIASIOLLI¹, M.F. DELBUT², A. DELL'ORCO¹, L. MALGARINI¹,
P. THEVENIN¹, J. VERNON², J. VIVANT², R. ZIMMERMANN¹ and G. GAY¹.

¹ Ciba Seeds, CH-4002 Basle, Switzerland.

² Département d'Amélioration des Plantes, INRA-Domaine d'Epoisses,
F-21110 Genlis, France

Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC₆ generation were obtained at the BC₃ generation, about one year after BC₁ seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure, if at all possible. Theoretically, a minimum

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1988) reported about 90% recurrent parent genotype recovery in two BC₁₀-equivalent conversions (A632Ht and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tanksley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgene construct) from a donor into a recipient maize line.

Materials and methods

Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgene construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Kozic *et al.* 1993). Transformation was achieved through microprojectile bombardment (Kozic *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

Backcross protocol

The F₁ progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC₁ progeny.

For each backcross generation, except the BC₄, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC₄ plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker

analyses. Results of marker analysis were available before flowering. A single plant was rescued and transferred onto tissue culture medium, before being grown in the field, on average, four months.

Molecular marker analysis

Restriction Fragment Length Polymorphism (RFLP) genotypes in all four genetic backgrounds were determined by chemiluminescent techniques. Loci were chosen from among those that provided coverage of the entire genome, contained two loci tightly linked to the gene of interest (within 10 recombination units away) (Figure 1). BC_{n+1} generation comprised both homozygous and heterozygous ones, and additional selected BC_n plant was heterozygous for the gene of interest in the independent reference population generation.

Selection procedure

At each generation plants of recurrent-parent-genotype and transgenic-parent-genotype were grown together. An attempt to integrate both criteria was made. Missing values were not included. The best ranking one of those for which the BC₃ selection was available was chosen.

Results and discussion

Selection for the gene of interest

The observed segregation was significantly different ($P < 0.05$) from the expected 1:1 ratio.

Recurrent parent genotype

Statistics for the genotype were performed taking the whole set of backcross-derived plants thereof.

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ations, to be achievable through the
al *et al.* 1992; Jarboc *et al.* 1994).
Genetic variability at each backcross
variability by applying the highest

investigated through an experiment
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origin was used as donor parent to
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projectile bombardment (Koziel *et*
chromosome 1 (Figure 1).

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Individuals were planted in multipots
carry the transgene construct. To
BC₄ plants carrying the transgene
with the *pat* and *Bt* genes. Resistant
leaf-sampled for molecular marker

analyses. Results of marker analyses were made available at the latest two weeks after
flowering. A single plant was selected, of which all backcross-derived embryos were
rescued and transferred onto tissue culture medium. Plantlets that developed from these
embryos first underwent a greenhouse acclimation phase, while still growing on tissue
culture medium, before being transplanted into multipots. Backcross cycles lasted, on
average, four months.

Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish
genotypes in all four generations. RFLP detection involved either radioactive or
chemiluminescent techniques. For the BC₁ generation, 61 marker-enzyme combinations
were chosen from among those revealing polymorphism between donor and recipient. They
provided coverage of the entire genome, defining intervals of about 25 cM in size, and
contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16
recombination units away (Figure 1). For subsequent generations, markers analyzed in the
BC_{n+1} generation comprised both those for which the selected BC_n plant was heterozygous,
or tightly linked ones, and additional ones located in chromosomal segments for which the
selected BC_n plant was heterozygous (Table 1). Marker map positions were obtained from
independent reference populations and confirmed by analysis of segregation in the BC₁
generation.

Selection procedure

At each generation plants were ranked based both on the percentage of homozygous
recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an
attempt to integrate both criteria. Plants for which two or more adjacent markers had
missing values were not included in the analyses. Success or failure of the pollinations also
contributed to the selection procedure. One single plant was selected at each generation: the
best ranking one of those for which a backcross progeny of size 100 or more (50 or more
for the BC₃ selection) was available.

Results and discussion

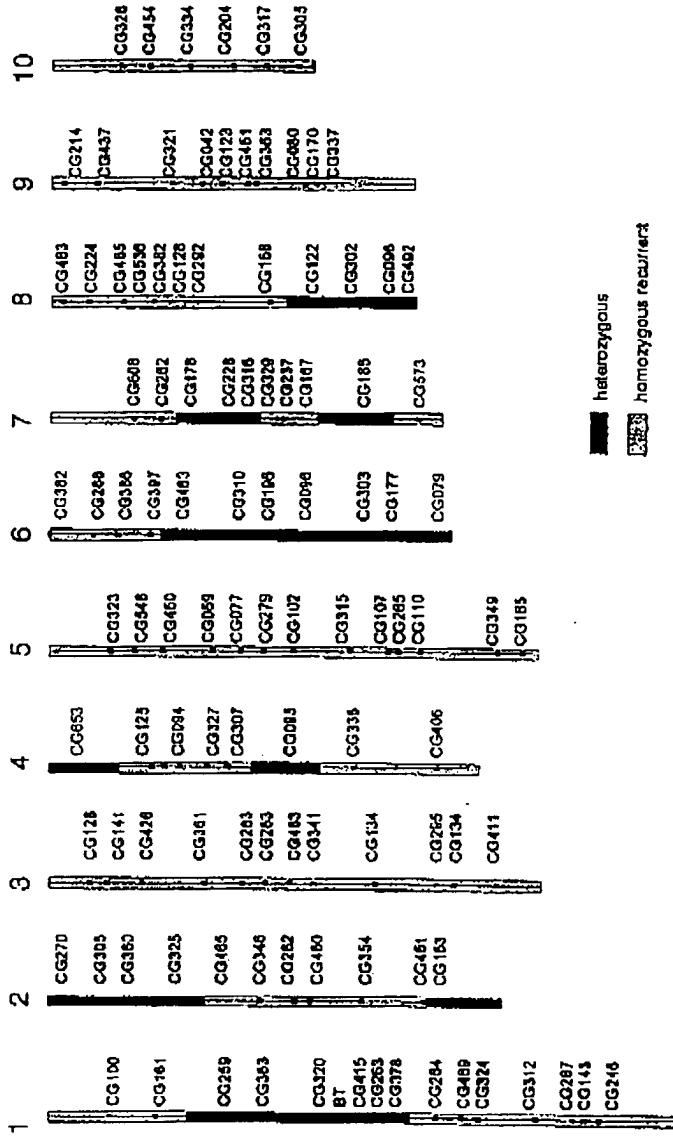
Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not
significantly different ($P < 0.05$) from the expected 1:1, as shown by Chi-square tests.

Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were
performed taking the whole genome into account, including the *Bt* locus. The "perfect"
backcross-derived plant therefore counts one heterozygous chromosome segment, that

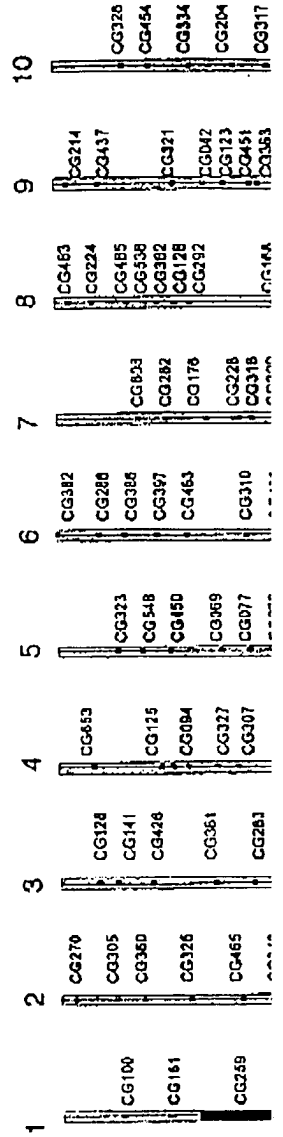
SELECTED BC1



heterozygous
homozygous recurrent

Figure 1-3: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

SELECTED BC2



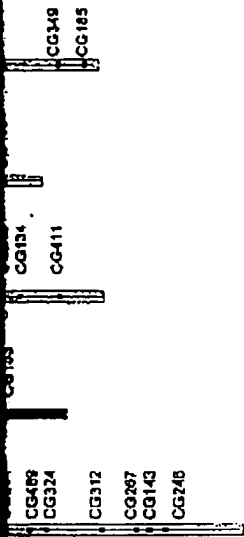


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

SELECTED BC2

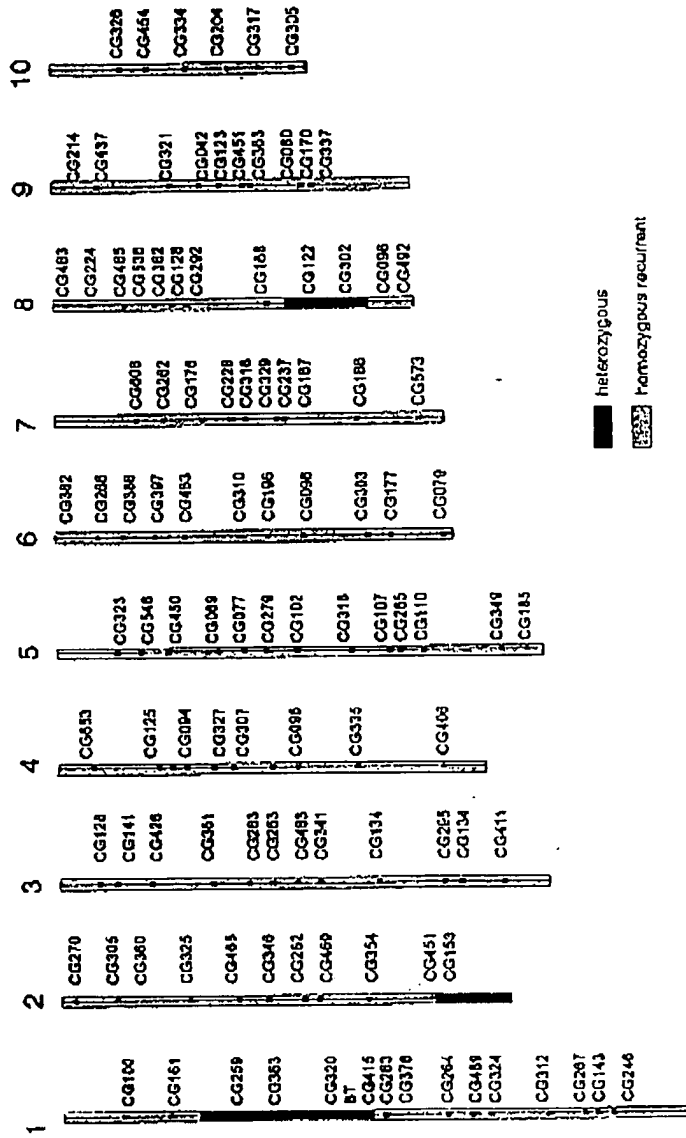
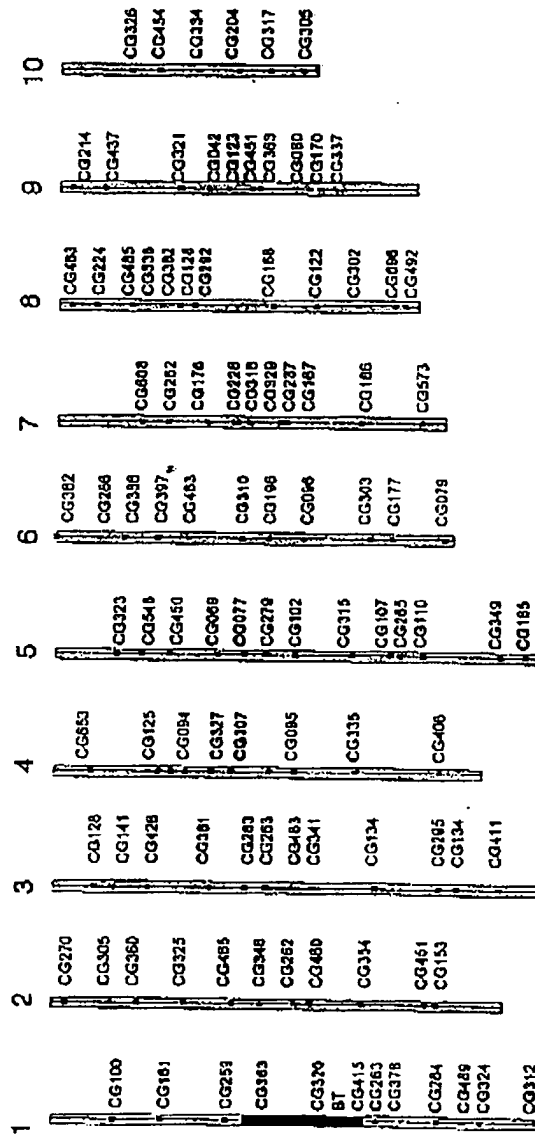


Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

SELECTED BC3

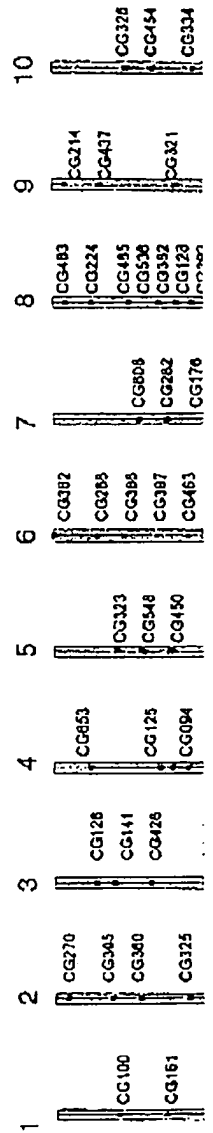


heterozygous

homozygous recurrent

Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

SELECTED BC4



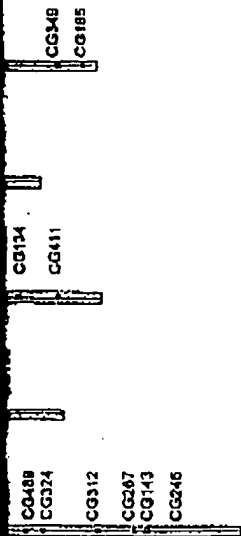


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

SELECTED BC4

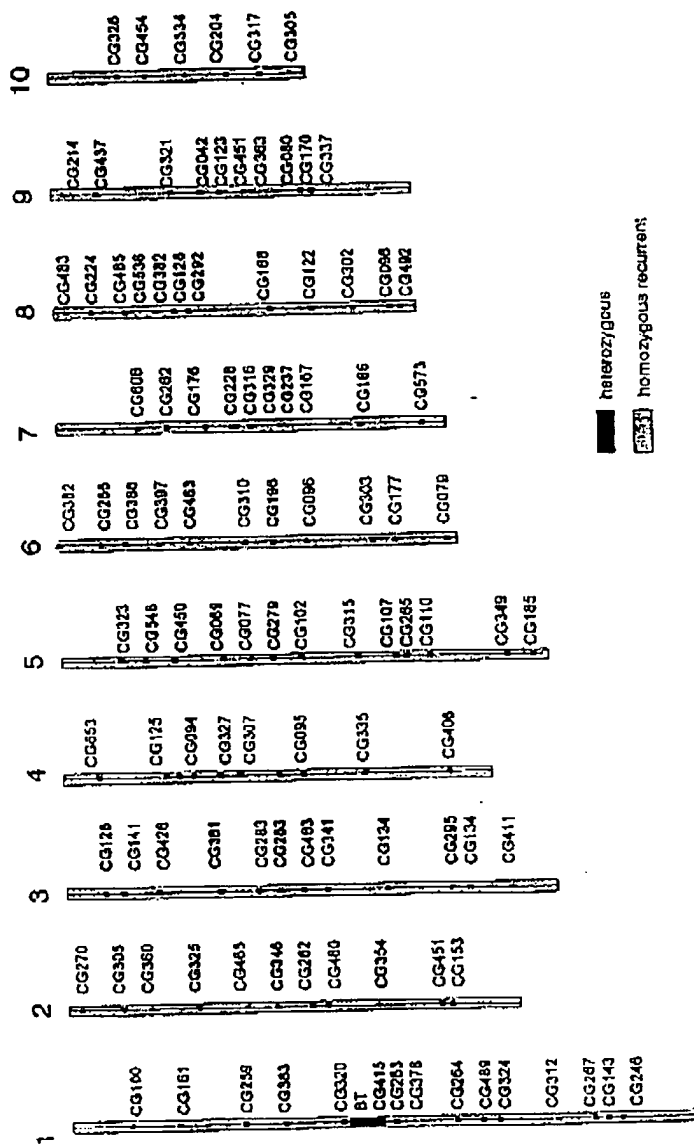


Figure 1-d: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

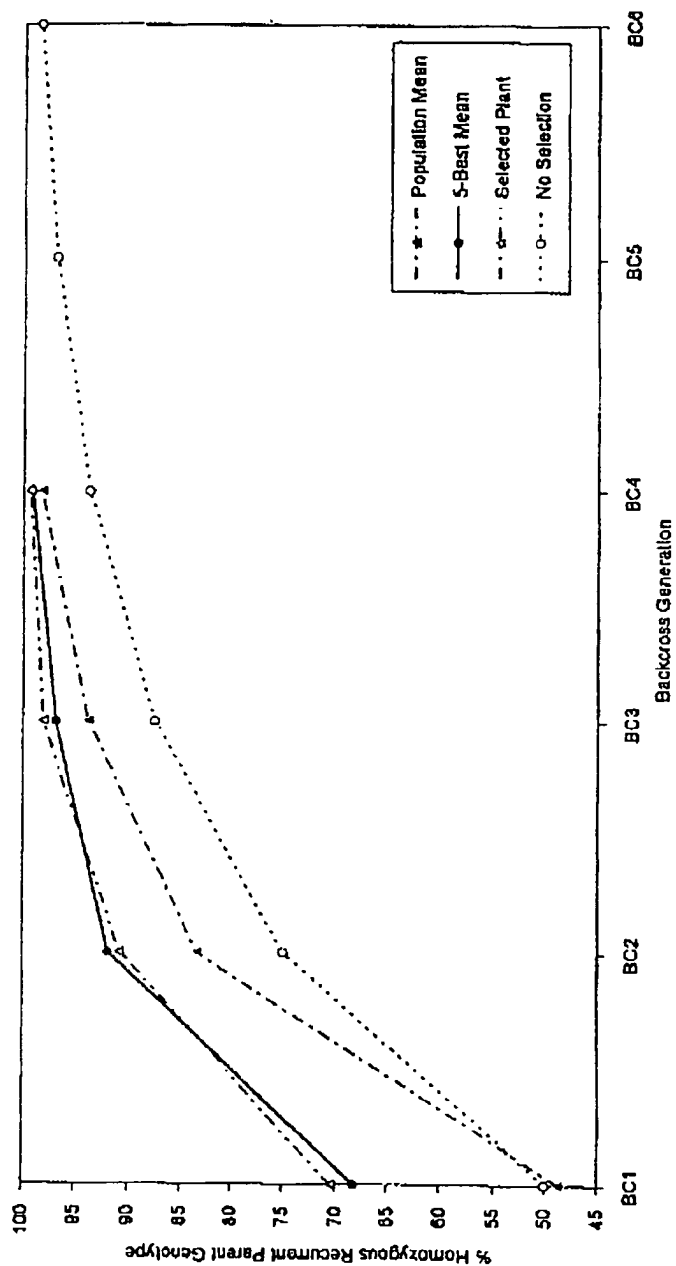


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

noneraine	% phoschithurcin	RFLP genotyping	nb plants	% homozygous recurrent	nb heterozygous
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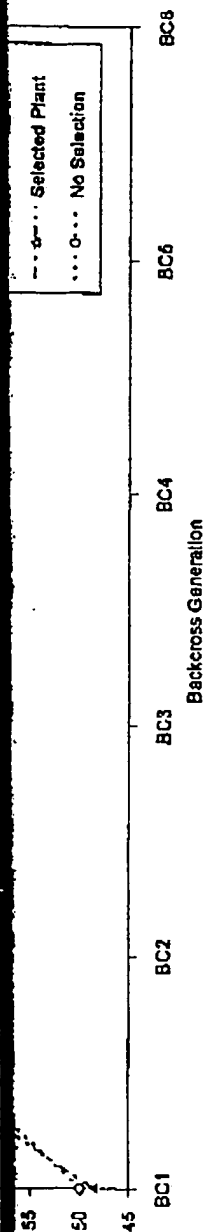


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype				nb heterozygous chromosome segments ***			
		nb plants	nb loci	nb datapoints		mean	std dev	5-best mean **	selected plant	mean	std dev	5-best mean **	selected plant
BC1	49.05	98	61	5856	87	48.72	10.35	89.31	70.45	11.01	2.17	7.75	8
BC2	44.65	61	22	1342	90	83.42	5.84	91.98	90.84	5.03	1.54	3.20	3
BC3	46.32	72	10	720	71	83.63	1.85	88.82	88.03	2.20	0.71	1.60	1
BC4	.	26	3	78	28	68.23	0.48	99.09	89.38	1.00	0.00	1.00	1

* Plants for which two or more adjacent markers had missing values were not included in the analyses.
 ** Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.
 *** Including the segment carrying the transgene construct.

comprising the *Bt* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Bt* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the BC₁ generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Bt* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Bt* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the BC₂ generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Bt* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected BC₁ plant was almost equal to that of an unselected BC₂, that of the selected BC₂ was larger than that of an unselected BC₃, that of the selected BC₃ was barely smaller than that of an unselected BC₆, and that of the selected BC₄ was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Jarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used, BC₃ and BC₄ plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Bt* locus.

Linkage drag

Linkage drag around the *Bt* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected BC₁ individual, between 17.6 and 34.8% for the selected BC₂, between 2.0 and 24.0% for the selected BC₃, and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected BC₄.

The two values given for each generation correspond to extreme positions of flanking the transgene construct locus. BC₄ is likely to be less than 1.3% appear to be somewhat high, reflecting linkage drag, it is much lower than what Stam and Zeven (1981; Tanksley *et al.* of tomato cultivars obtained by a linkage drag. Tanksley (1989) found that the sizes in cM.

Conclusion

These results clearly demonstrate the quality advantages over classical methods through backcrossing. Only four backcrosses, less than a year and a half from plant selection, genotypically fully converted. Nevertheless, the recurrent parent genotype could proceed even faster with an appropriate protocol and resources allocated.

Comparison of BC₄-derived lines with markers and agronomic performance in the field order to confirm the completeness of the conversion.

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homozygous recurrent-parent-genotype. The relative length of the chromosome between the two flanking markers chosen.

The parent-genotype of the BC₁ generation can be explained by linkage drag around the *Bt* locus based only on plants selected for backcross generations the mean percentage of recurrent parent genome was higher than what would have been expected (Figure 2).

The parent-genotype of the selected plant (Table 1) were always very similar to the expected value (Figure 2). The percentage of recurrent parent genome of the selected plant was found only once, in the five largest values. This corresponded to one with the maximum percentage of recurrent parent genome that had been selected because it displayed a high percentage of recurrent parent genome (Figure 1).

The parent-genotype of the selected BC₁ plant of the selected BC₂ was larger than that of the unselected BC₁ and was only slightly smaller than that of an unselected BC₁. The percentage of recurrent parent genome of the "perfect" backcross-derived BC₁ was higher than that of the unselected BC₁. The rates of recurrent parent genotype in the backcrosses were similar to those of Jarboe *et al.* (1994) who used backcross generations and 80 markers to estimate the percentage of recurrent parent genome.

The segments decreased from one backcross generation to the next. The segments were not necessarily those which were selected for the backcross. The segments recovered which contained only one *Bt* locus.

The percentage of recurrent parent genome relative to the length of chromosome was 14.5% for the selected BC₁ individual, between 2.0 and 24.0% for the selected BC₂ (14.5 cM) for the selected BC₄.

The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC₄ is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeven 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm-2* conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC₁'s, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC₄-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

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